THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 13, Issue of March 31, pp. 9876–9881, 2000

Printed in U.S.A.

Anchoring of Surface Proteins to the Cell Wall of *Staphylococcus aureus*

SORTASE CATALYZED *IN VITRO* TRANSPEPTIDATION REACTION USING LPXTG PEPTIDE AND NH_2-GLY_3 SUBSTRATES*

(Received for publication, December 7, 1999)

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Staphylococcus aureus **sortase anchors surface proteins to the cell wall envelope by cleaving polypeptides at the LPXTG motif. Surface proteins are linked to the peptidoglycan by an amide bond between the C-terminal carboxyl and the amino group of the pentaglycine cross-bridge. We find that purified recombinant sortase hydrolyzed peptides bearing an LPXTG motif at the peptide bond between threonine and glycine. In the presence of NH_2-Gly_3, sortase catalyzed exclusively a transpeptidation reaction, linking the carboxyl group of threonine to the amino group of NH_2-Gly_3. In the presence of amino group donors the rate of sortase mediated cleavage at the LPXTG motif was increased. Hydrolysis and transpeptidation required the sulfhydryl of cysteine 184, suggesting that sortase catalyzed the transpeptidation reaction of surface protein anchoring via the formation of a thioester acyl-enzyme intermediate.**

Surface proteins of *S. aureus* are covalently linked to the bacterial peptidoglycan by a mechanism requiring a C-terminal sorting signal (1, 2). The sorting signal is comprised of an LPXTG motif followed by a C-terminal hydrophobic domain and a tail of positively charged amino acids (3, 4). During cell wall sorting, surface proteins are cleaved between the threonine and the glycine of the LPXTG motif (5). The liberated carboxyl group of threonine is amide linked to the amino group of the pentaglycine cross-bridge (6), thereby tethering the C-terminal end of the polypeptide chain to the bacterial cell wall (7, 8). Surface protein cleavage at the LPXTG motif and peptidoglycan attachment occurs in staphylococcal protoplasts, *i.e.* bacteria in which the cell wall has been removed by digestion with muralytic enzyme (9). Vancomycin, an antibiotic that sequesters peptidoglycan precursors by binding to the D-Ala-D-Ala portion of lipid II (10), interferes with surface protein anchoring (9). Thus, surface proteins are likely linked to the pentaglycine cross-bridge of the lipid II peptidoglycan precursor. Lipid-linked intermediates are incorporated into the cell wall by the transpeptidation and transglycosylation reactions of bacterial cell wall synthesis (11).

Staphylococcus aureus strains carrying a knockout mutation in the sortase gene (*srtA*) fail to cleave sorting signals at the LPXTG motif, thereby abolishing cell wall anchoring and surface display of this class of proteins (12).[1] Sortase, a 206-amino acid polypeptide with an N-terminal signal sequence/stop transfer domain, is anchored in the cytoplasmic membrane of staphylococci.[1] Purified sortase as well as recombinant $SrtA_{\Delta N}$, an enzyme in which the N-terminal signal sequence has been removed, cleave LPXTG motif bearing peptides (14). The strong nucleophile hydroxylamine interferes with the cell wall sorting reaction of staphylococci (14). Although sorting signals are cleaved at the LPXTG motif, surface proteins containing a C-terminal threonine hydroxamate are released into the extracellular medium. Presumably, hydroxylamine attacks an acyl-enzyme intermediate between surface protein and sortase (14). When added to purified $SrtA_{\Delta N}$, hydroxylamine increases the overall rate of cleavage at the LPXTG motif. Both *in vitro* peptide cleavage and hydroxylaminolysis depend on cysteine 184 of sortase (14). We think it is likely that the cysteine sulfhydryl may function as the active site nucleophile to form a thioester bond between sortase and the carboxyl group of threonine at the C-terminal end of surface proteins. The principal components of the cell wall anchor structure of surface proteins[2] and the mechanism of protein attachment to the peptidoglycan are conserved in Gram-positive bacteria (16–19). Sortase (*srtA*) homologs have been found in the sequenced genomes of Gram-positive bacteria, all of which feature absolute conservation of the single cysteine sulfhydryl.[1]

Previous work left unresolved whether sortase catalyzes a transpeptidation reaction *in vitro* using the peptidoglycan cross-bridges as amino group donors. Furthermore, the site of sortase-mediated cleavage *in vitro* has thus far not been identified. We find that purified sortase performs peptide bond hydrolysis and transpeptidation by cleaving between the threonine and the glycine of the LPXTG motif. When incubated with the amino group nucleophile NH_2-Gly_3, sortase catalyzes exclusively the transpeptidation reaction. Thus, sortase functions as a transpeptidase to anchor surface proteins to the pentaglycine cross-bridge of the bacterial cell wall.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—S. aureus strains RN4220 and SKM1 (*srtA*$^-$) have been described previously (20).[1] Plasmid pSM34 was generated by polymerase chain reaction amplification from RN4220 chromosomal DNA using primers GSA1–12 (AAGGATCCTACCTTTTCCTCTAGCTGAAG) and SRT-C2A (CATTAATTACTGCTGATGATTAC) introducing a substitution of cysteine 184 with alanine. The

* This work was supported by United States Public Health Service Grant AI 38897. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] S. K. Mazmanian, G. Liu, E. R. Jensen, E. Lenoy, and O. Schneewind, submitted for publication.

[2] G. Dhar, K. F. Faull, and O. Schneewind, submitted for publication.

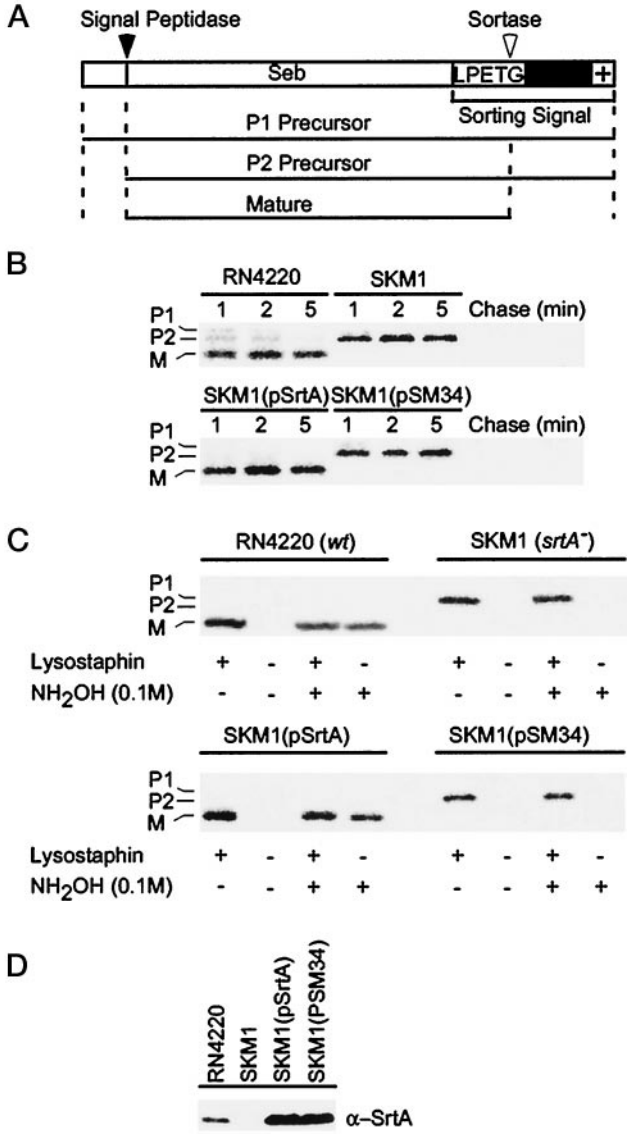

FIG. 1. **Cysteine 184 of sortase is required for surface protein hydroxylaminolysis and cell wall anchoring.** *A*, structure of Seb-Spa$_{490-524}$ harboring an N-terminal signal peptide and a C-terminal cell wall sorting signal consisting of the LP*X*TG motif, hydrophobic domain (*black box*), and positively charged tail (*boxed* +). P1 precursor is directed across the cytoplasmic membrane by the N-terminal signal peptide and cleaved to generate the P2 precursor. The sorting signal of P2 is cleaved at the LP*X*TG motif and the mature polypeptide (M) is linked to the bacterial cell wall. *B*, cell wall sorting of Seb-Spa$_{490-524}$ was followed by pulse labeling staphylococcal cultures. At the indicated time intervals, culture aliquots were precipitated with trichloroacetic acid and the cell wall was digested with lysostaphin. Seb-Spa$_{490-524}$ was immunoprecipitated with α-Seb, separated on 15% SDS-PAGE, and quantified by PhosphorImager analysis. Processing was analyzed in *S. aureus* RN4220, SKM1(*srtA*$^-$), SKM1(pSrtA, encoding wild-type SrtA), and SKM1(pSM34, encoding SrtA$_{C184A}$). *C,* staphylococcal cultures were pulse-labeled in the presence or absence of hydroxylamine. Trichloroacetic acid-precipitated samples were divided in two aliquots. One sample was boiled in SDS, whereas the other was subjected to peptidoglycan digestion prior to boiling in SDS. The addition of hydroxylamine caused *S. aureus* RN4220 to release pulse-labeled surface protein into the extracellular medium, whereas the sortase mutant strain SKM1 or SKM1 expressing SrtA$_{C184A}$ was unable to catalyze hydroxylaminolysis of surface proteins. *D*, immunoblotting of staphylococcal extracts with α-SrtA to detect the expression of sortase.

DNA fragment was purified and, together with the primer GSA1–5 (AAGGATCCAAAAGGAGCGGTATACATTGC), used for polymerase chain reaction amplification with RN4220 template DNA. The amplified DNA fragment was purified, digested with *Bam*HI, and ligated into pOS1 cut with *Bam*HI. Plasmids pSM34 and pSrtA were electroporated

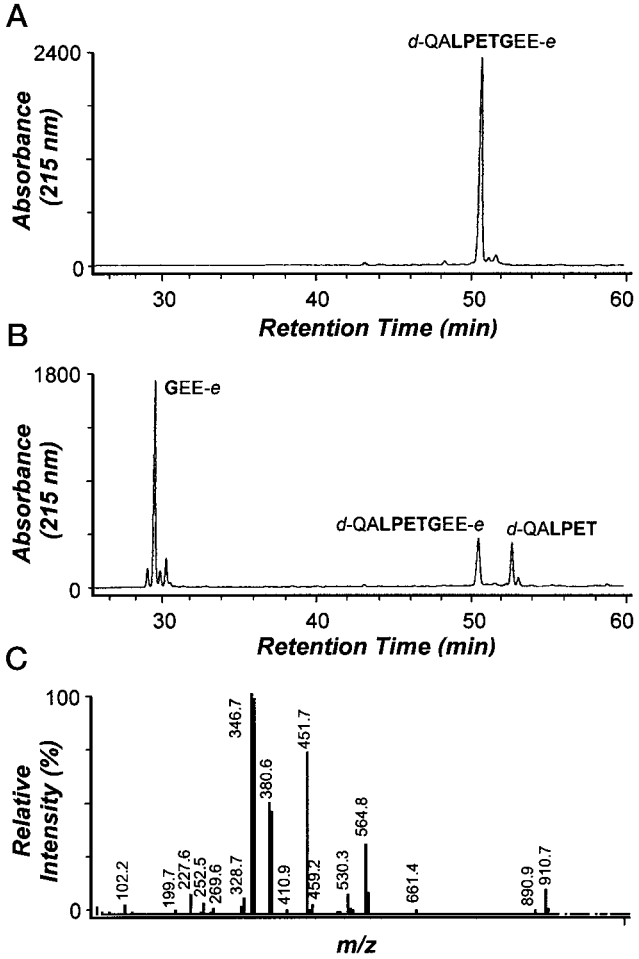

FIG. 2. **Purified sortase (SrtA$_{\Delta N}$) hydrolyses the peptide bond between threonine and glycine of the LP*X*TG motif.** *A*, RP-HPLC chromatogram of the substrate peptide *d*-QA**LPETG**EE-*e* on C-18 column (the LP*X*TG motif is printed in *bold*). *B*, *d*-QA**LPETG**EE-*e* was incubated with purified sortase (SrtA$_{\Delta N}$) at 37 °C for 16 h. The enzyme was removed by filtration and reaction products were separated by RP-HPLC. *C*, MS/MS of the RP-HPLC-purified peptide *d*-QA**LPET** (909 Da). collisionally induced dissociation of the singly charged parent ion at *m*/*z* 910.7 generated fragment ions that corroborated the predicted peptide sequence (see Table I).

into *S. aureus* SKM1 and transformants selected on chloramphenicol plates (10 μg/ml). Purification of recombinant sortase was performed as described previously (14).

Pulse-Chase Experiments—Staphylococcal cultures were grown overnight in chemically defined medium, diluted 1:20 into minimal medium and pulse-labeled at A_{600} of 0.5. One ml of culture was labeled with 100 μCi of [^{35}S]Promix (Amersham Pharmacia Biotech) for 1 min and the incorporation of radioactive amino acids into polypeptides was quenched by the addition of 50 μl of chase solution (100 mg/ml casamino acids, 10 mg/ml methionine and cysteine). Cultures were incubated and at defined intervals 250-μl aliquots were withdrawn and precipitated with trichloroacetic acid. Precipitates were digested with lysostaphin (1 ml of 0.5 M Tris-HCl, pH 8.0, 100 μg/ml enzyme) for 1 h at 37 °C. Digests were precipitated by adding 75 μl of 100% trichloroacetic acid, centrifuged, washed in acetone, and dried. The precipitate was solubilized by boiling in hot SDS and subjected to immunoprecipitation.

Hydroxylaminolysis of Staphylococcal Surface Proteins—Staphylococci were grown in minimal medium until A_{600} 0.5. Four reaction tubes were prepared that contained either 100 μl of 0.5 M Tris-HCl, pH 7.5, or 100 μl 0.5 M Tris-HCl, pH 7.5, and 0.1 M hydroxylamine. To each tube, 1 ml of culture (10^9 cells) was added and bacteria were pulse-labeled with 100 μCi of Promix for 1 min. After the addition of chase solution (50 μl of 100 mg/ml casamino acids, 20 mg/ml methionine and cysteine), cells were incubated at 37 °C for 5 min. All reactions were precipitated with trichloroacetic acid and suspended in 1 ml of 0.5 M Tris-HCl, pH 7.0. Where indicated, peptidoglycan was digested with 100 μg of lysostaphin for 1 h at 37 °C. Samples were again precipitated with trichlo-

TABLE I
Summary of daughter ions produced during MS/MS of the 909 Da compound

Observed	Charge state	Calculated[a]	Δobs-calc[b]	Proposed structure	Ion type[c]
m/z		*m/z*			
102.2	+1	103.1	−0.9	T	z_1
199.7	+1	199.2	+0.5	QA	—[d]
227.6	+1	226.2	+1.4	PE	—[d]
252.5	+1	252.3	+0.2	Dabcyl	b_0
269.6	+1	267.3	+2.3	Dabcyl	c_0
328.7	+1	327.3	+1.4	PET	—[d]
346.7	+1	346.4	+0.3	PET	y_3
380.6	+1	380.4	+0.2	Dabcyl-Q	b_1
410.9	+1	409.5	+1.4	QALP	—[d]
451.7	+1	451.5	+0.2	Dabcyl-QA	b_2
459.2	+1	459.5	−0.3	LPET	y_4
530.3	+1	530.6	−0.3	ALPET	y_5
564.8	+1	564.7	+0.1	Dabcyl-QAL	b_3
661.4	+1	661.8	−0.4	Dabcyl-QALP	b_4
890.9	+1	892.0	−1.1	Dabcyl-QALPET	b_6
910.7	+1	910.0	+0.7	Dabcyl-QALPET	Parent ion

[a] Calculations are based on average masses according to the MacBioSpec™ program.
[b] The difference between the observed and calculated masses of daughter ions.
[c] Nomenclature refers to N- and C-terminal cleavage fragments according to Roepfstorff and Fohlman (15) and Biemann (13).
[d] Fragments thought to arise by two cleavages are calculated as the sum of the residue masses.

roacetic acid, washed in acetone, dried, and then boiled in SDS. Aliquots were subjected to immunoprecipitation with α-Seb[3] and analyzed by SDS-PAGE and PhosphorImager.

Immunoprecipitation—Samples were boiled in SDS (100 μl of 4% SDS, 0.5 M Tris-HCl, pH 8.0), centrifuged for 5 min at 15,000 × *g* and the supernatant was transferred to a new tube. Twenty μl of sample was immunoprecipitated with α-Seb diluted 1:1000 into RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxylcholate, 0.1% SDS, pH 7.5) and collected on protein A-Sepharose beads. The beads were washed five times in RIPA buffer and boiled in 50 μl of sample buffer prior to separation on 15% SDS-PAGE.

Kinetic Analysis of Recombinant Sortase—Dabcyl-QALPETGEE-*Edans* (*d*-QALPETGEE-*e*) was dissolved in 20% dimethyl sulfoxide and added to the kinetic reaction at a final concentration between 1 and 6 μM. Peptide cleavage was monitored as an increase of fluorescence using a FluoroMax-2 spectrometer (Instruments S.A., Inc.). The peptide was incubated in the presence or absence of 5 mM amino group nucleophile and 4.71 μM $SrtA_{\Delta N}$ in buffer R (150 mM NaCl, 5 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.5) in a volume of 520 μl. The increase in fluorescence intensity was recorded as a function of time using excitation at 350 nm and recording the emission maximum at 495 nm. Initial velocities were calculated as units of fluorescence per unit time using the following equation,

$$V_0 = m^*[S]/(I_{100} - I_0) \quad \text{(Eq. 1)}$$

where m is a slope during the linear phase of the cleavage, [S] is substrate concentration, and I_0 and I_{100} are the fluorescence intensities of substrate solution before and after complete cleavage (21). Slope (m) was measured in three independent experiments. Kinetic constants K_m, V_{max}, and k_{cat} were calculated from the curve fit for the Michaelis-Menten equation using the Lineweaver-Burk plot (22).

HPLC Purification of Cleaved Products—A reaction mixture consisting of 10 μM fluorescent peptides, 15 μM recombinant enzymes in 520 μl of Buffer R was incubated either in the presence or absence of 5 mM NH_2-Gly_3 at 37 °C for 16 h. The reaction was quenched by filtration using Centricon-10 filters (Millipore). The filtrate was subjected to RP-HPLC purification on C-18 column (2 × 250-mm, C18 Hypersil, Keystone Scientific). Separation was carried out at 40 °C with a gradient from 1 to 41% CH_3CN (0.1% trifluoroacetic acid) in 41 min and from 41 to 100% for 10 min at a flow rate of 0.2 ml/min. Elution of peptides was monitored at 215 nm and fractions were collected every minute, dried under vacuum, and stored at 4 °C for ESI-MS analysis as described previously (7, 23).

RESULTS

Hydroxylaminolysis of Staphylococcal Surface Proteins Requires the Cysteine Residue of Sortase—If cysteine 184 functions as a nucleophile for the cell wall anchoring reaction, a sortase enzyme lacking this sulfhydryl should be unable to cleave surface proteins at the LP*X*TG motif. This assumption was tested by replacing cysteine 184 of sortase with alanine ($SrtA_{C184A}$). Plasmids encoding either wild-type (pSrtA) or mutant sortase (pSM34) were transformed into *S. aureus* SKM1 (*srtA*$^-$). Staphylococci were examined for the ability to process cell wall sorting signals. Wild-type staphylococci synthesize surface protein precursor bearing an N-terminal signal peptide and a C-terminal sorting signal (P1 precursor). Following export across the cytoplasmic membrane and signal peptide cleavage, sortase cleaves the P2 precursor between the threonine and the glycine of the LP*X*TG motif to generate mature, cell wall anchored surface protein (Fig. 1*A*). As expected, the sortase mutant strain SKM1 failed to cleave polypeptides at the LP*X*TG motif and accumulated P2 precursor species (Fig. 1*B*). Transformation of SKM1 cells with plasmids that allowed expression of wild-type SrtA, but not $SrtA_{C184A}$, restored precursor processing at the LP*X*TG motif. Thus, the sulfhydryl of cysteine 184 is absolutely necessary for the processing of sorting signals and the cell wall anchoring of surface proteins.

We asked whether cysteine 184 of sortase is required for the formation of a sortase acyl-enzyme intermediate and examined the hydroxylaminolysis of surface proteins *in vivo*. When pulse-labeled staphylococcal cultures are precipitated with trichloroacetic acid and boiled in SDS, only proteins secreted into the extracellular medium are soluble in hot SDS (3). In contrast, staphylococcal surface proteins, membrane or cytoplasmic proteins require digestion of the cell wall with lysostaphin for solubility in hot SDS (3). Staphylococcal cultures were pulse-labeled in the presence or absence of hydroxylamine. Trichloroacetic acid-precipitated samples were divided in two aliquots. One sample was boiled in SDS, whereas the other was subjected to peptidoglycan digestion prior to boiling in SDS. The addition of hydroxylamine caused wild-type staphylococci to release pulse-labeled surface protein into the extracellular medium (Fig. 1*C*). The sortase mutant strain SKM1 was unable to catalyze hydroxylaminolysis and all pulse-labeled surface protein precursors required digestion of the staphylococcal cell wall for solubility in hot SDS. Transformation of strain SKM1 with plasmids that expressed wild-type SrtA, but not $SrtA_{C184A}$, restored hydroxylaminolysis of surface proteins (Fig. 1, *C* and *D*). Similar to *srtA*$^-$ cells, the cysteine mutant accumulated uncleaved P2 precursor species that required lysostaphin digestion of the peptidoglycan for solubility in hot SDS. Thus, the sulfhydryl of cysteine 184 is absolutely required for the formation of sortase acyl-enzyme intermediates.

In Vitro Hydrolysis of LPXTG Bearing Peptides—Purified $SrtA_{\Delta N}$ was used to study *in vitro* hydrolysis and transpeptidation reactions of sortase. To determine the site of *in vitro* substrate cleavage, *d*-QALPETGEE-*e* peptides were incubated with $SrtA_{\Delta N}$. Enzymatic reactions were quenched by filtration, separating the enzyme from peptide substrate and products. Sample filtrate was analyzed by RP-HPLC. When incubated without $SrtA_{\Delta N}$, *d*-QALPETGEE-*e* eluted as a single absorption peak at 50 min (36% CH_3CN) (Fig. 2*A*). However, after incubation of *d*-QALPETGEE-*e* with sortase, two new peaks

[3] The abbreviations used are: Seb, staphylococcal enterotoxin B; Dabcyl, 4-(4-dimethylaminophenyl-azo)benzoic acid; *d*-QALPETGEE-*e*, Dabcyl-QALPETGEE-Edans; Edans, 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid; ESI-MS, electrospray ionization mass spectrometry; MS/MS, tandem mass spectrometry; Ni-NTA, nickel-nitriloacetic acid; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reverse phase-high performance liquid chromatography; PBP, penicillin-binding protein.

were identified that eluted at 29 (15% CH_3CN) and 53 (39% CH_3CN) min (Fig. 2*B*). Collected RP-HPLC fractions were analyzed by ESI-MS. Measurements were consistent with the cleavage of *d*-QALPETGEE-*e* (observed mass 1471.8, calculated mass 1471.6) between the threonine and the glycine of the LP*X*TG motif, generating the cleavage products *d*-QALPET (observed mass 908.4, calculated mass 909.0) and GEE-*e* (observed mass 581.1, calculated mass 580.6) (data not shown). To further characterize the reaction products, the 581.1-Da peptide was subjected to Edman degradation, yielding the peptide sequence GEE. The 908.4-Da peptide was analyzed in an MS/MS experiment (Fig. 2*C*). Collisionally induced dissociation of the parent ion at m/z 910.7 generated fragment ions that confirmed the compound structure *d*-QALPET (Table I). Together these results demonstrate that sortase hydrolyses the peptide bond between the threonine and the glycine of the LP*X*TG motif.

In Vitro Transpeptidation of LPXTG Bearing Peptides—We sought to determine whether sortase catalyzes a transpeptidation reaction in the presence of the physiological nucleophile, *i.e.* the amino group of glycine. The presumed peptidoglycan substrate of the sorting reaction, lipid II (undecaprenyl-pyrophosphate-MurNac(-L-Ala-D-iGln-L-Lys(NH_2-Gly_5)-D-Ala-D-(Ala)-β1–4-GlcNac)), is difficult to purify in sufficient quantities (11). We therefore replaced lipid II with NH_2-Gly_3 as a peptidoglycan substrate. The polypeptide substrate *d*-QALPETGEE-*e* and NH_2-Gly_3 were incubated with $SrtA_{\Delta N}$, reaction products were filtered and analyzed by RP-HPLC. In addition to the residual substrate peaks at 50 min (*d*-QALPETGEE-*e*) and 4 min (NH_2-Gly_3) (data not shown), two new peaks of absorption at 215 nm were identified (Fig. 3, *A* and *B*). The compound that eluted at 29 min was analyzed by ESI-MS and generated an average mass of 581.1 Da, consistent with the predicted mass of the peptide GEE-*e*. This result indicated that sortase had cleaved *d*-QALPETGEE-*e* at the peptide bond between threonine and glycine. The compound that eluted at 51 min was also analyzed by ESI-MS, producing ion signals at m/z 540.8 and 1080.8 with an average compound mass of 1079.7. These measurements are consistent with the calculated mass of the transpeptidation product *d*-QALPET-Gly_3 (1080.2 Da). To determine the structure of this compound, the singly charged parent ion at m/z 1080.8 was analyzed in an MS/MS experiment (Fig. 3*C*). Collisionally induced dissociation produced ions confirmed the peptide sequence *d*-QALPET-Gly_3 (Table II). The chromatograms of Fig. 2*B* and Fig. 3*A* were enlarged to more closely examine the product peaks (Fig. 3*B*). When sortase was incubated with *d*-QALPETGEE-*e* and NH_2-Gly_3, no hydrolysis product (*d*-QALPET) could be detected. Thus, sortase catalyzed exclusively the transpeptidation reaction and accumulated only the products *d*-QALPET-Gly_3 and GEE-*e*.

Kinetic Measurements of Sortase Catalyzed Hydrolysis and Transpeptidation—Fluorescence of the Edans fluorophore (*e*) within the peptide *d*-QALPETGEE-*e* is quenched by the close proximity of Dabcyl (*d*). When the peptide is cleaved by sortase, and the fluorophore is separated from Dabcyl, an increase in fluorescence is observed. During transpeptidation conditions, *i.e.* when $SrtA_{\Delta N}$ was incubated with *d*-QALPETGEE-*e* and NH_2-Gly_3, the rate of peptide cleavage was increased as compared with the rate of hydrolysis at the LP*X*TG motif (*i.e.* incubation of $SrtA_{\Delta N}$ with *d*-QALPETGEE-*e* but without NH_2-Gly_3) (Fig. 4, *a* and *b*). As a control, incubation of *d*-QALPETGEE-*e* with a cysteine mutant enzyme, $SrtA_{\Delta N,\ C184A}$, did not result in substrate cleavage between the threonine and the glycine of the LP*X*TG motif (Fig. 4, *d*). Furthermore, the addition of the sufhydryl reagent [2-(trimethylammonium)ethyl-

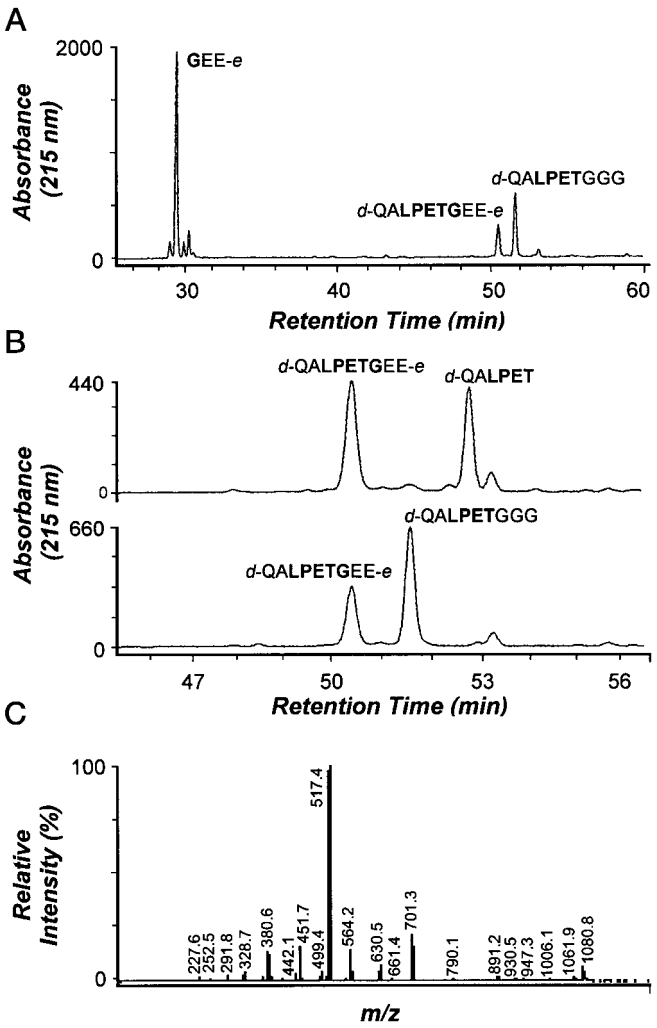

FIG. 3. **Purified sortase ($SrtA_{\Delta N}$) catalyzes the transpeptidation reaction of surface protein anchoring *in vitro*.** *A*, *d*-Q**ALPETG**EE-*e* substrate peptide was incubated with $SrtA_{\Delta N}$ in the presence of 5 mM NH_2-Gly_3 at 37 °C for 16 h. The enzyme was removed by filtration and reaction products were separated by RP-HPLC. *B*, enlargement of RP-HPLC chromatograms comparing sortase-catalyzed hydrolysis (Fig. 2*B*) and transpeptidation products (*A*). *C*, MS/MS of the RP-HPLC-purified 1080 Da peptide (*d*-Q**ALPET**-Gly_3). Collisionally induced dissociation of the singly charged parent ion at m/z 1080.8 generated fragment ions that corroborated the predicted peptide sequence (see Table II).

]methanethiosulfonate inhibited sortase and abolished all peptide cleavage (Fig. 4*c*). Together these results indicate that sortase catalyzes the transpeptidation reaction at a rate that is at least 2-fold faster than the rate of peptide bond hydrolysis (Table III). As expected, the sulfhydryl of cysteine 184 is absolutely necessary for *in vitro* substrate cleavage to occur. A K_m of 16.48 μM and a K_{cat} of 2.27×10^{-5} (1/s) was calculated for the sortase-catalyzed transpeptidation reaction (Table III). The affinity of sortase for *d*-QALPETGEE-*e* substrate was slightly increased in the presence of NH_2-Gly_3 as was the overall efficiency of the cleavage reaction. Immediately after mixing reaction components, $SrtA_{\Delta N}$ did not cleave substrate for several minutes. We do not know the reason for this delay in cleavage activity.

We asked whether the rate of substrate cleavage depended on the nature of the added nucleophile. Addition of the strong nucleophile hydroxylamine caused a relatively small increase in the rate of *d*-QALPETGEE-*e* cleavage (Table IV). However, the presence of the weaker nucleophiles NH_2-Gly, NH_2-Gly_2, or NH_2-Gly_3 caused a greater increase in the rate of substrate

TABLE II
Summary of daughter ions produced during MS/MS of the 1080 Da compound

Observed	Charge state	Calculated[a]	Δobs-calc[b]	Proposed structure	Ion type[c]
m/z		*m/z*			
227.6	+1	226.2	+1.4	PE	—[d]
252.5	+1	252.3	+0.2	Dabcyl	b_0
291.8	+1	291.3	+0.5	TGGG	y_4
328.7	+1	327.3	+1.4	PET	—[d]
380.6	+1	380.4	+0.2	Dabcyl-Q	b_1
442.1	+1	441.4	+0.7	PETGG	—[d]
451.7	+1	451.5	+0.2	Dabcyl-QA	b_2
499.4	+1	500.5	−1.1	PETGGG	y_6-OH
517.4	+1	517.5	−0.1	PETGGG	y_6
564.2	+1	564.7	−0.5	Dabcyl-QAL	b_3
630.5	+1	630.7	−0.2	LPETGGG	y_7
661.4	+1	661.8	−0.4	Dabcyl-QALP	b_4
683.6	+1	684.7	−1.1	ALPETGGG	z_8
701.3	+1	701.8	−0.5	ALPETGGG	y_8
790.1	+1	790.9	−0.8	Dabcyl-QALPE	b_5
891.2	+1	892.0	−0.8	Dabcyl-QALPET	b_6
930.5	+1	932.0	−1.5	Dabcyl-QALPETG	b_7-OH
947.3	+1	949.0	−1.7	Dabcyl-QALPETG	b_7
1006.1	+1	1006.1	0	Dabcyl-QALPETGG	b_8
1061.9	+1	1063.1	−1.2	Dabcyl-QALPETGGG	b_9
1080.8	+1	1081.2	−0.4	Dabcyl-QALPETGGG	parent ion

[a] Calculations are based on average masses according to the MacBioSpec™ program.
[b] The difference between the observed and calculated masses of daughter ions.
[c] Nomenclature refers to N- and C-terminal cleavage fragments according to Roepfstorff and Fohlman (15) and Biemann (13).
[d] Fragments thought to arise by two cleavages are calculated as the sum of the residue masses.

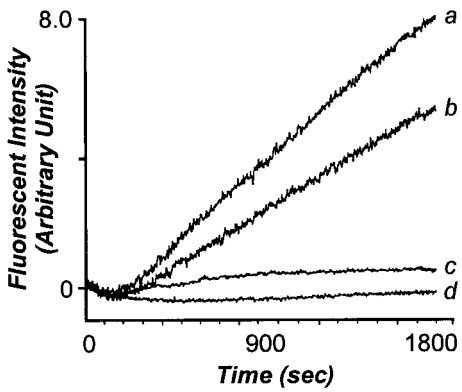

FIG. 4. **Kinetic analysis of sortase-catalyzed hydrolysis and transpeptidation reactions.** $SrtA_{\Delta N}$ catalyzes a transpeptidation reaction in the presence of 5 mM NH_2-Gly_3 (*a*) at a faster rate than hydrolysis of the substrate peptide *d*-QALPETGEE-*e* in the absence of amino group nucleophiles (*b*). These reaction are inhibited by 5 mM [2-(trimethylammonium)ethyl]methanethiosulfonate, a sulfhydryl reagent (*c*). $SrtA_{\Delta N,\ C184A}$, carrying a substitution of cysteine 184 with alanine, failed to cleave the substrate peptide (*d*). Reaction mixtures contained 5.2 μM *d*-QALPETGEE-*e*, 4.7 μM $SrtA_{\Delta N}$, or $SrtA_{\Delta N,\ C184A}$ in 520 μl of buffer R. Reactions were incubated in the presence or absence of 5 μM NH_2-Gly_3 at 37 °C for 30 min.

cleavage, suggesting that sortase recognizes the physiological nucleophile of the transpeptidation reaction, *i.e.* the amino group of the pentaglycine cross-bridge (Table IV). The fastest rate of substrate cleavage was observed when sortase was incubated in the presence of NH_2-Gly_3.

DISCUSSION

Surface protein anchoring in *S. aureus* is catalyzed via a transpeptidation reaction, employing polypeptide and peptidoglycan substrates (2). The polypeptide substrate, *i.e.* surface protein precursor with C-terminal sorting signal bearing an LP*X*TG motif, is cleaved by sortase between the threonine and the glycine of the LP*X*TG motif (5). As pulse-labeled surface proteins cannot be found in the extracellular medium, sortase does not hydrolyze polypeptides at the LP*X*TG motif *in vivo* (3). Cleaved polypeptides are thought to be captured as acyl-enzyme intermediates, presumably involving cysteine 184 and the formation of a thioester bond (14). The intermediate is resolved by the nucleophilic attack of the cross-bridge amino group, resulting in the formation of an amide bond between the threonine of the LP*X*TG motif and the pentaglycine cross-bridge (6). We think it is likely that lipid II serves as a peptidoglycan substrate for the cell wall sorting reaction (9). However, as lipid II biosynthesis is essential for staphylococcal growth, this prediction cannot be tested with isogenic pairs of wild-type and mutant strains that fail to synthesize lipid II. Our work is therefore focused on the biochemical characterization of the sorting reaction and we demonstrate here that purified sortase catalyzes a transpeptidation reaction at the LP*X*TG motif. The rate of sortase mediated cleavage at the LP*X*TG motif is increased in the presence NH_2-Gly, NH_2-Gly_2, or NH_2-Gly_3, suggesting that the enzyme interacts with the cell wall cross-bridge. Furthermore, these results suggest that the rate-limiting step of surface protein anchoring is the nucleophilic attack of the acyl-enzyme intermediate. This hypothesis may also explain the relative resistance of sortase to various slow-reacting sulfhydryl reagents: the active site sulfhydryl of sortase is generally engaged with an acyl intermediate of cleaved surface protein (9).

During cell wall synthesis, bacterial penicillin-binding proteins (PBPs) cleave glycan-linked cell wall pentapeptides (L-Ala-D-iGln-L-Lys-D-Ala-D-Ala) at the amide bond between D-Ala-D-Ala (24, 25). The carboxyl group of D-Ala is ester linked to the enzyme active site, *i.e.* the hydroxyl group of serine (26). Nucleophilic attack of the amino group of glycine resolves the enzyme intermediate, forming an amide bond between D-Ala and the pentaglycine cross-bridge of neighboring peptidoglycan strands (27). Kozarich and Strominger (28) have purified and studied the 46-kDa PBP from *S. aureus* H, using diacetyl-L-Lys-D-Ala-D-Ala and NH_2-Gly as substrates. In the absence of amino group nucleophiles, the *S. aureus* enzyme hydrolyzed the amide bond between D-Ala-D-Ala (28). However, at NH_2-Gly

TABLE III
Kinetic analysis of $SrtA_{\Delta N}$

Kinetic constants K_m, V_{max}, and k_{cat} were calculated from the curve fit for the Michaelis-Menten equation using the Lineweaver-Burk plot. Reaction conditions are described in the legend to Fig. 4.

Nucleophile	K_m	V_{max}	K_{cat}	K_m/K_{cat}
	μM	μM/s	l/s	l/μM · s
H_2O	10.88	5.08×10^{-5}	1.06×10^{-5}	9.77×10^{-7}
NH_2-Gly_3	16.48	1.08×10^{-4}	2.27×10^{-5}	1.38×10^{-6}

TABLE IV
The effect of different nucleophiles on the rate of LPXTG peptide cleavage by sortase ($SrtA_{\Delta N}$)

Nucleophile	M (s^{-1})[a]
H_2O	1.84 (±0.11)
NH_2OH	1.91 (±0.07)
NH_2-Gly	1.95 (±0.05)
NH_2-Gly_2	2.03 (±0.13)
NH_2-Gly_3	2.91 (±0.03)

[a] Slope of the kinetic curves as shown in Fig. 4. The substrate peptide *d*-QALPETGEE-e was incubated with $SrtA_{\Delta N}$ and various nucleophiles. Substrate cleavage between the threonine and the glycine was measured as an increase in fluorescence. With the exception of water, all nucleophiles were added at a concentration of 5 mM. Averages were calculated from three independent experiments and standard deviations are reported (parentheses).

concentrations of 1 mM or higher this PBP performed exclusively the transpeptidation reaction of bacterial cell wall synthesis. The addition of glycine increased the overall performance of the *S. aureus* enzyme as measured by the cleavage of diacetyl-L-Lys-D-Ala-D-Ala (28). Hydroxylamine, although a stronger nucleophile than glycine (29), caused a much smaller increase in enzyme performance than glycine, while the addition of D-Ala inhibited the transpeptidase activity (28). A V_{max} of 0.4 μM/min/mg enzyme and substrate K_m 100 mM were observed for the *S. aureus* PBP as compared with the V_{max} of 0.06 μM/min/mg enzyme and K_m 16.48 μM described here for sortase. The results of Kozarich and Strominger (28) suggest that the *S. aureus* PBP specifically binds the amino group nucleophile glycine and that this recognition event is a rate-limiting step in the overall transpeptidation reaction. Thus, the staphylococcal PBP displays properties that are similar to those observed for sortase.

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